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AS RELATED TO PLANETARY QUARANTINE

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INTRODUCTION

This report covers research activities during the period December 1, 1968, through May 31, 1969, on the project Environmental Microbiology as Related to Planetary Quarantine, an activity of the Division of Environmental Health, School of Public Health, University of Minnesota, and the National Aeronautics and Space Administration.

In the body of this report, the progress made in each of the project areas is described. Since the common thread of dry heat resistance of microorganisms as a function of temperature runs through several of these project areas, it seems appropriate to tie this material together in the Introduction to the report.

In each of the several studies in this project, the destruction of dry spores appears to be related directly to spore water content or to parameters which affect spore water content. Although the research data all point to the critical nature of the water in and surrounding the spore, we have thus far been unable to correlate the effect of this water in one experiment with its effect in other experiments. It is imperative that a thorough understanding of the role of water in dry spore destruction be developed. As a start toward developing this understanding, three questions which have grown out of our research will be presented and discussed. The questions are as follows:

1. What is the true effect of the quantity of water in the spore at heating medium temperature on the D-value?
2. What is the effect of the quantity of water in the spore at the start of heating when the spores are heated in

an open system in which most of the water initially present in the spore will be lost in the first few minutes of heating?

3. What is the relationship of the water content and D-value at 125°C to the water content and D-value at 45°C?

Each of these questions will be treated separately below.

1. What is the true effect of the quantity of water in the spore at heating medium temperature on the D-value?

Angelotti et al. (1968)[a] reported D-values as a function of the relative humidity at which spores were equilibrated on non-polymerized plastic at 25°C prior to encapsulation of the spores in a plastic matrix. This is certainly the best data available to us today; however, it has the limitation that it is for microorganisms encapsulated in plastic. The moisture content of the encapsulated microorganisms is the moisture content in equilibrium with a specified relative humidity at 25°C, assuming that the plastic matrix is neither a source nor a sink of water.

In an attempt to develop a relationship for open systems, we show in Figure 1, a logarithm of the D-value versus the logarithm of relative humidity (measured at the test temperature) of the gas in contact with the spores during heating. These data are from Silverman (1968).[b] (We have assumed that his casual atmosphere had a relative humidity of

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[a] Angelotti, R. A., J. H. Maryanski, T. F. Butler, J. T. Peeler and J. E. Campbell, 1968. Influence of spore moisture content on the dry-heat resistance of Bacillus subtilis var. niger. Appl. Microbiol., 16(5), pp. 735-745.

[b] Silverman, G., 1968. The resistivity of microorganisms to inactivation by dry heat. Report submitted to NASA under Contract NsG-691. Massachusetts Institute of Technology, Cambridge, Massachusetts.

0.7% and that the dry air had a relative humidity of 0.1% at the test temperature.)

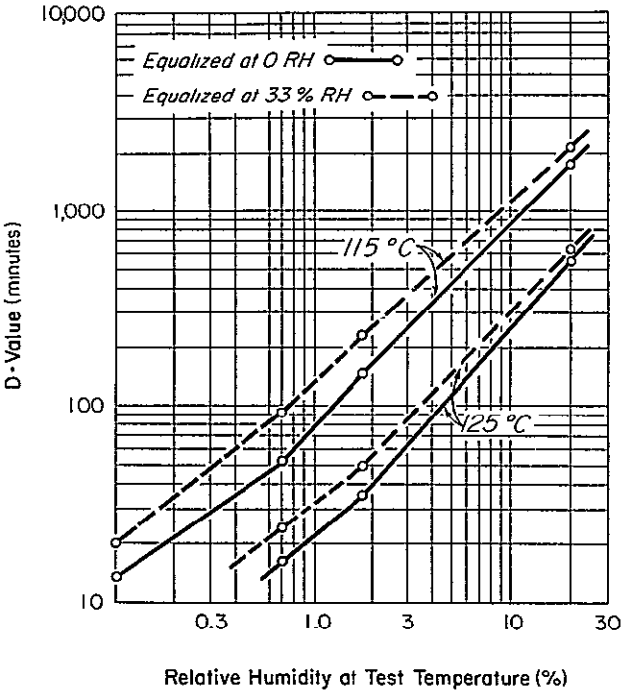

FIGURE 1

D-values of Bacillus subtilis var. niger spores as a function of the relative humidity at test temperature.

It seems probable that there is a single relationship between spore D-value and spore water content. When we have arrived at this single relationship, it will correlate the data for dry heat destruction of microorganisms on open surfaces, microorganisms in mated surface areas and microorganisms encapsulated in solids. This must be true since the spore itself will never know whether it is encapsulated, in a mated surface area or on an exposed surface; all it will see is its internal condition which will result from the external location condition. The relationship reported by Angelotti et al. (1968)[a] and the relationship we show in Figure

[a] Angelotti, R. A., J. H. Maryanski, T. F. Butler, J. T. Peeler and J. E. Campbell, 1968. op cit.

l, therefore, must be somehow combined. The importance of developing this relationship cannot be overstressed because an explanation of what happens in a specific area can only be developed if the basic D-value – water content relationship of the spore is known.

2. What is the effect of the quantity of water in the spore at the start of heating when the spores are heated in an open system in which most of the water initially present in the spore will be lost in the first few minutes of heating?

The data regarding the effect of water on the dry heat destruction of microbial spores which are available at this time suggest that the water content of the spore at the start of heating and the water content of the spore during heating may have independent effects on the D-value. Dry heat experiments carried out by Silverman (1968)[a], Hoffman et al. (1968)[b] and Drummond and Pflug (1969)[c] in constant humidity atmospheres yielded D-values that varied with the relative humidity of the atmosphere in which the spores were equilibrated. D-values increased when the relative humidity during equilibration increased. The experiments of Silverman (1968)[d] and Drummond and Pflug (1969)[e] were also carried out at several

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[a] Silverman, G., 1968. op cit.

[b] Hoffman, R. K., V. M. Gambill and L. M. Buchanan, 1968. Effect of cell moisture on the thermal inactivation rate of bacterial spores. Appl. Microbiol., 16(8), pp. 1240-1244.

[c] Drummond, D. W. and I. J. Pflug, 1969. Effects of humidity on the dry heat destruction rate of Bacillus subtilis var. niger spores on surfaces. Abstract A48, Bact. Proc. 1969. Paper presented at 69th Annual Meeting of the American Society for Microbiology, May 4 through 9, 1969, Miami Beach, Florida.

[d] Silverman, G., 1968. op cit.

[e] Drummond, D. W. and I. J. Pflug, 1969. op cit.

treatment humidity levels; Drummond and Pflug (1969)[a] evaluated very low humidities while Silverman (1968)[b] evaluated relative humidities up to 20%.

Angelotti et al. (1968)[c] and Campbell (1969)[d] have both reported that dry microbial cells gain or lose water very rapidly. Their data suggest that the equilibrium is established in a matter of minutes after exposure to the new environment.

If microbial cells are equilibrated in a chamber at 33% relative humidity and 22°C, they will contain more water than cells equilibrated at 11% relative humidity and 22°C. If both groups of cells are subsequently heated in an atmosphere with a relative humidity of 0.1% at 115°C, the cells will lose water. If the test is carried out at a temperature where the D-value is several hours and the total heating period extends until the microbial population has been reduced from 10^6 to 10^3 spores per sample, then the majority of the heating time will occur after the major amount of water loss which, at the new temperature and humidity condition, will occur in the first hour of heating.

Water loss from microbial cells during dry heat testing is a form of drying; therefore, we can expect the water content of the cells to decrease exponentially. The drying curves of the cells equilibrated at 33% and 11% should be parallel. If the instantaneous D-value were a function only of the instantaneous quantity of water in the spore, then

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[a] Drummond, D. W. and I. J. Pflug, 1969. op cit.

[b] Silverman, G., 1968. op cit.

[c] Angelotti, R. A., J. H. Maryanski, T. F. Butler, J. T. Peeler and J. E. Campbell, 1968. op cit.

[d] Campbell, J. E., 1969. Quarterly Progress Report #16 (April). Environmental Control Administration, Public Health Service, Cincinnati, Ohio.

the survivor curve for 33% and 11% relative humidity should be parallel and the D-value equal.

Since Silverman (1968)[a], Hoffman et al. (1968)[b] and Drummond and Pflug (1969)[c] found that the D-values varied with the relative humidity at which the cells were equilibrated, then one of the premises above must be wrong. Either the spore water content curves are not parallel, the initial water content affects the subsequent rate of water loss, or the initial water content has an effect that is separate and different from the effect of the water content of the spores during the heating period. The existing experimental data on rate of moisture loss tend to refute the idea that drying rates are different. Is it possible that the quantity of water in the spore at the time it is heated to the sterilization temperature determines the configuration of some critical molecule inside the cell?

3. What is the relationship of the water content and D-value at 125°C to the water content and D-value at 45°C?

Dry heat destruction rate data in the temperature range 115 to 135°C indicate that as spore moisture content decreases, the destruction rate increases. The effect of drying on spore survival at high temperatures appears to be opposite to the effect of drying on spore survival at low temperatures. The standard method of preserving microorganisms is to lyophilize the culture and then store the lyophilized culture at re-

[a] Silverman, G., 1969. op cit.

[b] Hoffman, R. K., V. M. Gambill and L. M. Buchanan, 1968. op cit.

[c] Drummond, D. W. and I. J. Pflug, 1969. op cit.

frigerator temperatures.

Microbial survival studies carried out at 22 and 45°C indicate that longer survival occurs at these temperatures when the spores are very dry. Spores in a 10% relative humidity atmosphere show a very small reduction in numbers in a six to eight week period whereas spores in a 50% relative humidity atmosphere show a rapid die-off.

These results suggest that somewhere between 45 and 125°C there are some interesting changes in microbial survival patterns and perhaps in macromolecular stability. Work underway in this project will hopefully illuminate the factors which are causing these changes.

I. J. Pflug

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SURVIVAL OF BACILLUS SUBTILIS VAR. NIGER SPORES AT TEMPERATURES BELOW 60°C

Project Personnel: D. Vesley, G. Smith and J. Haugen - Division of Environmental Health

Project Contributors: Eugene Johnson - Division of Biometry

INTRODUCTION

In Progress Report #1 (December, 1968) the background and general experimental procedure to be followed in this study were described along with the method for achieving controlled humidity conditions in sealed plastic containers. The results of the first series of experiments to determine survival rates of B. subtilis var. niger spores at 22 and 45°C under <10%, ∿50% and >90% relative humidity conditions were reported. The work on this project has been continued during the present reporting period.

OBJECTIVE

The objective of these experiments has been to determine the effect of temperature and humidity on the long range survival of Bacillus subtilis var. niger spores. Both a closed and an open system as well as various temperature and humidity conditions are being evaluated. These data may be useful in controlling the contamination level on spacecraft prior to the terminal sterilization cycle.

EXPERIMENTAL PROCEDURE

Experiment I. In Progress Report #1 the experimental procedure for a three week study of the effect of temperature and humidity on the survival of B. subtilis var. niger spores was described. A 28 week study

has been initiated using the same conditions and procedures and another condition has been added by placing inoculated strips, contaminated side up, in the laminar downflow clean room at 22°C, ∿30%RH and 90 fpm velocity. The clean room strips were analyzed in a manner identical with the other strips. Raw data are available for 0, 1, 2, 3, 4, 8, 12, 16, 20, 24 and 28 weeks. Statistical analysis of the data has been completed through 20 weeks.

Experiment 2. A separate 12 week experiment has been initiated to fill in data on survival of B. subtilis var. niger spores at 45°C between <10% and 50%RH. The experimental procedure is identical to Experiment 1 except for the relative humidity conditions and the time intervals for analyses. The conditions being studied are 45°C and <10%, ∿25%, ∿35% and ∿50%RH. Time intervals for analysis are 0, 2, 4, 6, 10 and 12 weeks. Analyses through six weeks have been completed.

RESULTS AND DISCUSSION

Experiment 1. The range of humidity values actually recorded for each experimental condition is reported in Table 1.1. Relative humidity measurements of each temperature-humidity condition were made each time strips were assayed.

TABLE 1.1

Relative Humidity Ranges Recorded

Desired %RH	Recorded %RH	
	22°C	45°C
<10	<2	<2-9
∿30	30-40	--
∿50	50-59	55- 57
>90	88-96	96-100

The number of microorganisms surviving per strip as a function of time for each condition studied are shown graphically in Figure 1.1. D-values for each test condition are listed in Table 1.2. The D-values were calculated in the following manner: the best linear fit to the data in the form of logarithms of survivors as a function of time for each condition was calculated using the method of least squares. The slope of the resulting line was in turn converted into D-values.

TABLE 1.2

D-values of Bacillus subtilis var. niger Spores at Several Temperature-Humidity Conditions. (20 week data from 28 week experiment)

Temperature °C	%RH	D-value (days)
22	<10	388.9
	∿30(LFR*)	109.4
	∿50	291.7
	>90	66.0
45	<10	39.1
	∿50	7.4
	>90	4.0

* Laminar Flow Room

It can be observed in Figure 1.1 that at 45°C the spore survival rate decreased as the humidity increased; the D-value was 4.0 days for >90%RH and 7.4 days for ∿50%RH. No spores were recovered after four weeks at 45°C and >90%RH, after eight weeks at 45°C and ∿50%RH, and after 28 weeks at 45°C and <10%RH. At 45°C and <10%RH the calculated D-value was 39.1 days. It is interesting to note that with a D-value of 39.1 days, 28 weeks are required for the microbial population to decrease from 10^5 to one microorganism.

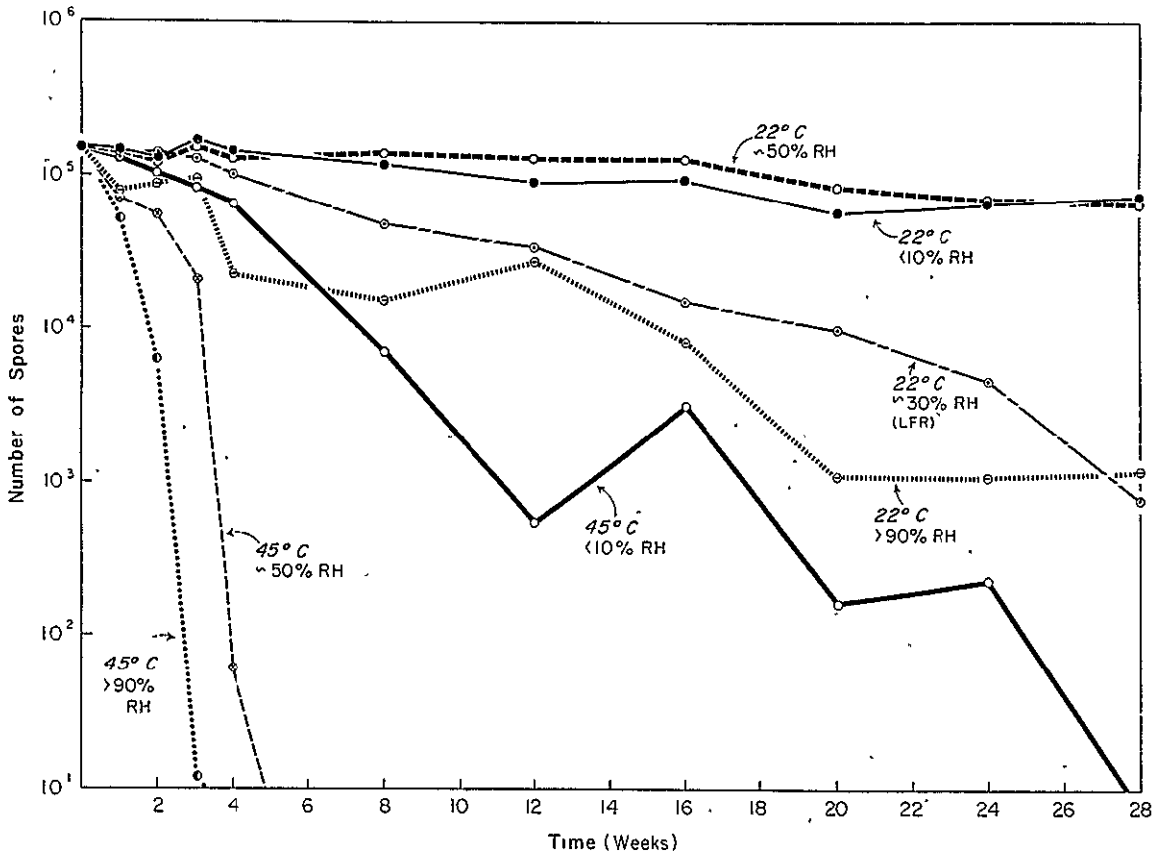

FIGURE 1.1

Survival of Bacillus subtilis var. niger spores as a function of time at several controlled temperature and humidity conditions.

On those occasions when no growth was observed after the incubation sequence, additional attempts were made to recover spores which may have been injured by the storage condition but not destroyed. These attempts included extended incubation (up to two weeks) and heat shock of the eluate and strip at 80°C for 20 minutes and 60°C for 60 minutes. In addition, several special media and germinating agents were utilized. The special media included Dextrose Starch Broth, Tryptone Glucose Yeast Extract Broth, and Trypticase Soy Broth. The germinating agents, added after heat shock, included L-alanine (100 µg/ml) and Insonine. None of these techniques resulted in additional spore recovery.

Speculation as to the mechanism for spore destruction under the conditions tested suggests that germination is probably not involved in the destruction processes. Previous investigations indicate that germination will not occur in an unsaturated environment (Beers, 1957)[a]. Thus it is more likely that a process resembling dry heat destruction is occurring and progressing more rapidly at high than at low humidity.

The D-values at 22°C are much longer than those at 45°C and the general survival pattern is quite different. At 22°C with an RH of <10% or ∿50%, the decrease in the number of survivors is small compared to about 99% reduction when the RH was >90%. There appears to be something unique about the behavior of spores in the laminar flow room (22°C, ∿30%RH) where the population decreased by about 90% in the 20 weeks. The only difference between the controlled humidity boxes and the laminar flow room would appear to be the air movement.

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[a] Beers, K. J., 1957. Effect of moisture activity on germination. Spores I, H. O. Halvorson, editor, American Institute of the Biological Sciences, Washington, D.C., pp. 45-51.

Experiment 2. Data have been collected for the first six weeks of the twelve week experiment at 45°C and <10%, ∿25%, ∿35% and ∿50%RH. The data are summarized in Figure 1.2 and Table 1.3.

TABLE 1.3

Survival of Bacillus subtilis var. niger Spores at 45°C and Various Relative Humidities. (6 week data from 12 week experiment.)

RH	Number of Survivors per Strip x 10^2			
	0 Time	2 Weeks	4 Weeks	6 Weeks
<10	2550.	1980.	978.	28.9
∿25	2550.	1630.	889.	12.8
∿35	2550.	1630.	1180.	60.6
∿50	2550.	547.	.546	<.01

The results of the <10% and ∿50%RH conditions follow the same trend as the results of the 28 week experiment shown in Figure 1.1. However, of particular interest are the results of the ∿25% and ∿35% relative humidities which coincide very closely with the results of the <10% condition which indicate that the spores subjected to <10%, ∿25% or ∿35%RH survive at a similar rate but at ∿50%RH the survival rate is very much different. Since the relative humidity in the atmosphere surrounding the spore determines the water content of the spore, it seems probable that the water content in equilibration somewhere between 35% and 50% is a critical water content level; above this level a more rapid microbial die-off takes place. This may be further clarified by the results of the remaining six weeks of this study.

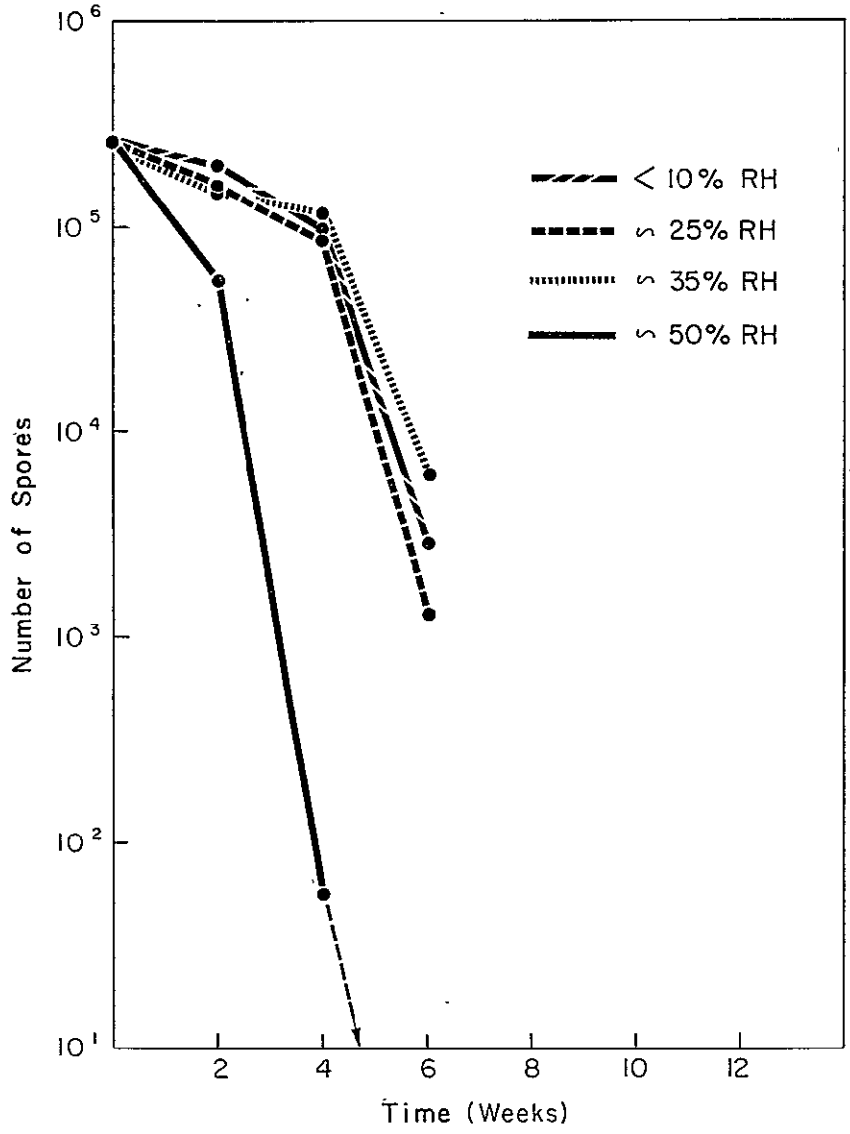

FIGURE I.2

Survival of Bacillus subtilis var. niger spores as a function of time for four relative humidity conditions at 45°C.

CONCLUSIONS

The die-off rate of B. subtilis var. niger spores is relatively rapid at ∿50% and >90%RH at 45°C and may have potential utility in reducing the microbial loading for a terminal sterilization cycle.

FUTURE WORK

The research underway will be continued and research will be initiated to determine more specifically the effect of the temperature-humidity interaction on the survival of bacterial spores. Three additional approaches are suggested.

1. Studies on the survival of B. subtilis var. niger have been conducted on spores pipetted onto surfaces from alcohol or distilled water suspensions. It is suggested that we conduct similar studies on spores falling out onto surfaces following aerosolization.
2. Studies to date have been conducted using B. subtilis var. niger spores. It is proposed to determine whether similar effects will be noted for other spore forming species. It is suggested that an anaerobic species (from the genus Clostridium) be tested in a manner similar to B. subtilis.
3. Studies of spore survival under specific temperature and humidity conditions have been conducted at 22 and 45°C. Previous studies have indicated a preservative effect at temperatures below 22°C. It is proposed to evaluate survival of Bacillus subtilis var. niger spores at approximately 60°C and <10%, ∿50% and >90% relative humidities.

This information should help to complete knowledge of spore survival generated by dry heat D-value determinations at temperatures above 60°C.

SURVIVAL OF BACILLUS SUBTILIS VAR. NIGER SPORES IN A CONTROLLED AIR STREAM

Project Personnel: George A. Carson and I. J. Pflug - Division of Environmental Health

INTRODUCTION

This is a study to determine the effect of air stream velocity on the survival of Bacillus subtilis var. niger spores at near ambient temperatures. The spores are deposited on stainless steel surfaces and air is blown over the inoculated surfaces. The December, 1968, Progress Report covered survival studies made at ambient temperature (25°C) and for velocities of 220, 745 and 1450 feet per minute (fpm).

OBJECTIVE

The objective of this project is to develop D-values based on the three parameters - time, temperature and velocity - for use in the design of NASA spacecraft sterilization procedures and to gain further insight into the effect of air velocity on the survival of B. subtilis var. niger spores.

EXPERIMENTAL PROCEDURE

The experimental procedure and apparatus used are the same as that described in Report #1. However, since the last reporting period an air heating device has been attached to make experimental runs at elevated temperatures. Figure 2.1 shows the stainless steel duct with the heater inside. It also shows how the duct is attached to the intake of the blower and the damper to control the air flow.

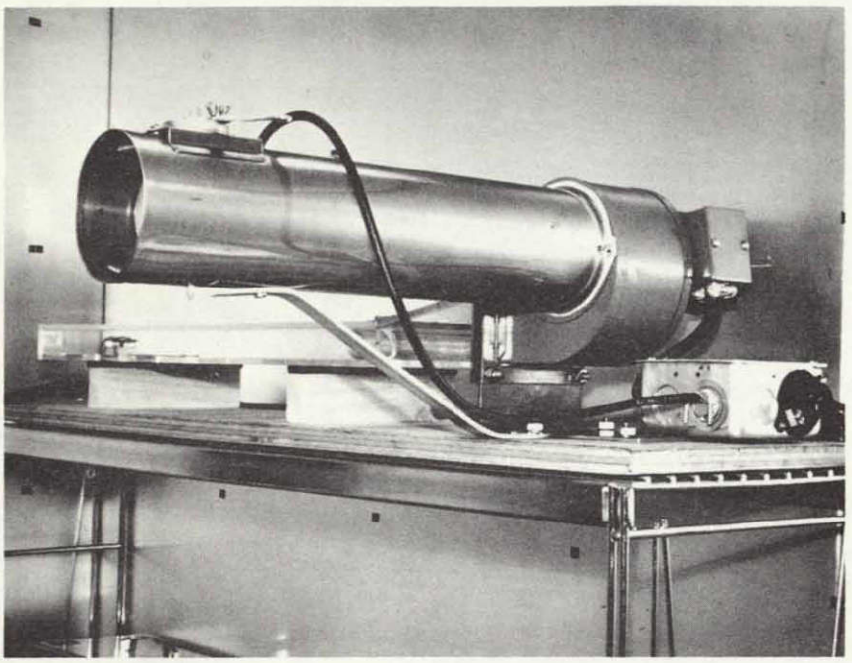

FIGURE 2.1

Air heating device attached to plexiglass duct.

As was mentioned in the data analysis from the previous report, the velocity of 220 fpm appeared to have the greatest effect. The exact reason for this is not known. One way to explain this phenomenon is in terms of the velocity boundary thickness. Assuming the flow in the duct is similar to the flow across a flat plate, it was determined that the critical velocity was around 150 fpm. Below this velocity the flow was laminar and above it turbulent.

With this information it was then decided to make a run at 100 fpm (laminar flow) at an elevated temperature (45°C). This run along with two others (100 fpm, 25°C and 750 fpm, 45°C) would give a block of data which could be analyzed for the effect of temperature and velocity.

TABLE 2.1

Summary of Experimental Runs

Run Number	Temperature °C	Velocity fpm	Flow Conditions
1	25	745	turbulent
2	25	1450	turbulent
3	25	220	turbulent
4	45	100	laminar
5	45	750	turbulent
6	25	100	laminar

RESULTS AND DISCUSSION

The results from the last reporting period indicated that there was no position effect. However, in the recent runs the strips were still removed from the duct in a manner which would allow the examination of the position effect. This was done primarily so that all the runs would be subjected to identical treatments.

Tables 2.2, 2.3, and 2.4 display the data for three runs (#4, 5 and 6). The runs were made in a vertical laminar flow room using the ambient air as supply air. The relative humidity of the room is 45% ±5% and the temperature is 25°C. The relative humidity of the air heated to 45°C is approximately 10%.

Each of the numbers shown at the top of the tables under "Latin Square" are the average of duplicate plate counts. The total number of organisms may be obtained by multiplying by 500.

In computing the Analysis of Variance Table the two plate counts were combined and treated as separate entities. Previously these plate

TABLE 2.2

Summary of Data

Run number 4 — Temp 45°C
Velocity 100 fpm — RH 10%

LATIN SQUARE

		Column						Sum
		1		2		3		
Week	1	B157	B211	A170	A191	C162	C179	1070
	2	A205	A178	C176	C184	B181	B154	1078
	3	C178	C163	B163	B169	A232	A176	1081
	Sum	1092		1053		1084		3229

	A	B	C
Sum	1152	1035	1042

ANALYSIS OF VARIANCE

Source of Variation	df	S.S.	M.S.	F
Rows	2	22	11	<1
Columns	2	283	142	<1
Time	2	2871	1436	1.56
Error	2	1840	920	
Total	8	5016	--	--

AVERAGE AND COEFFICIENT OF VARIATION OF COUNTS

		Controls		Exposed	
		Avg	CoV	Avg	CoV
Initial		291	2.6%	--	--
Row	A	220	18.7%	191	12.2%
	B	212	7.8%	172	12.2%
	C	216	6.9%	173	5.2%

TABLE 2.3

Summary of Data

Run number 5 — Temp. 45°C
Velocity 750 fpm — RH 10%

LATIN SQUARE

		Column						Sum
		1		2		3		
Row	1	B315	B349	A231	A345	C271	C232	1743
	2	A364	A369	C308	C276	B327	B330	1974
	3	C322	C278	B350	B322	A274	A337	1883
Sum		1997		1832		1771		5000

	A	B	C
Sum	1920	1993	1687

ANALYSIS OF VARIANCE

Source of Variation	df	S.S.	M.S.	F
Rows	2	9,027	4514	10.4
Columns	2	9,114	4557	10.5
Time	2	17,028	8514	19.6
Error	2	867	434	
Total	8	36,036	--	--

AVERAGE AND COEFFICIENT OF VARIATION OF COUNTS

		Controls		Exposed	
		Avg	CoV	Avg	CoV
Initial		379	3.8%	--	--
Week	A	312	15.7%	320	17.2%
	B	350	6.3%	332	4.2%
	C	318	11.3%	281	11.2%

TABLE 2.4

Summary of Data

Run number 6
Velocity 100 fpm

Temp. 25°C
RH 45%

LATIN SQUARE

		Column						Sum
		1		2		3		
Row	1	B 48	B 50	A 89	A 72	C 33	C 40	332
	2	A 90	A 78	C 36	C 26	B 49	B 51	330
	3	C 24	C 40	B 54	B 76	A 88	A 88	370
Sum		330		353		349		1032

	A	B	C
Sum	505	328	199

ANALYSIS OF VARIANCE

Source of Variation	df	S.S	M.S.	F
Rows	2	338	169	<1
Columns	2	101	51	<1
Time	2	15,734	7867	40.8
Error	2	385	193	
Total	8	16,558	--	--

AVERAGE AND COEFFICIENT OF VARIATION COUNTS

		Controls		Exposed	
		Avg	CoV	Avg	CoV
Initial		132	12.1%	--	--
Week	A	147	8.4%	84	8.6%
	B	107	14.1%	55	19.2%
	C	90	17.4%	33	20.9%

counts were put into duplicate Latin Squares but for this analysis they were combined into one. For example, in Table 2.2, the data for position, Row 1, Column 1, is 157 + 211 = 368. This figure, along with the others, was then used in the computation of the Analysis of Variance Table.

An F-test was performed on the data to determine the significance of the position effect as compared to the inherent error in the data. A significant F at the 5% level for 2 x 2 degrees of freedom is 19.00. The F-value for the position effect is not significant for any of the runs. However, for Runs 5 (45°C, 750 fpm) and 6 (25°C, 100 fpm), the F-test for time is significant. In other words, the spores undergo a significant die-off with time under these treatment conditions.

Table 2.5 shows the difference between the controls and the treated strips for four runs. The values were obtained by subtracting the average at the end of a treatment period of the six treated strips from the control strips. A negative sign indicates that the exposed plate count was greater than the control.

TABLE 2.5

Comparison of Plate Counts for Four Runs
Average of 6 controls - Average of 6 exposed strips

Velocity fpm	Treatment Period	Temperature	
		25°C	45°C
100	1 week	63	29
	2 weeks	52	40
	3 weeks	57	43
750	1 week	14	- 8
	2 weeks	- 3	18
	3 weeks	35	37

Looking at the table qualitatively it can be said that the 100 fpm velocity has the greatest effect. As mentioned previously, the 100 fpm velocity is laminar flow and the 750 fpm is turbulent flow; whether this phenomenon is causing the difference is not known but it may be possible.

According to these results, the temperature does not appear to have an effect. These temperatures are low and it may be necessary to run the experiment for longer periods of time before a temperature effect is noted.

FUTURE WORK

Future work will be directed at further analysis of the data to determine if the experiment warrants further study.

BEHAVIOR OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES

Project Personnel: D. Vesley and G. Smith - Division of Environmental Health

INTRODUCTION

The results of previous experiments regarding the effect of various conditioning factors on microbial spore D-values indicate that conditioning factors which affect the spore moisture content affect the spore D-value. The critical nature of the spore water content requires standardized conditions for equilibrating and handling spore samples. Activities during the past six months have been a continuation of the activities described in Report #1. These efforts are all designed to establish factors that contribute to variation so that our results yield reproducible D-values in relation to various physical conditions.

OBJECTIVE

The objective of the experiments reported here was to determine D-values of *B. subtilis* var. *niger* spore crops which have different known histories and to study the effect of different quantities of water in a closed system.

EXPERIMENTAL PROCEDURE

1. Support material. The spores were supported on 1" x 2" stainless steel strips, type 302, full hard, 0.014" thick. The strips were prepared by:

a. washing in nonionic detergent,

b. rinsing five times in tap water,

c. rinsing twice in distilled water,

d. dipping in isopropyl alcohol,

e. dipping in ethyl ether,

f. drain drying, and

g. sterilizing in a dry heat oven in petri dishes.

2. Spores. Spore history was the variable being studied in these experiments. B. subtilis var. niger spores from two sources were utilized. One spore crop was received from the Public Health Service Communicable Disease Center Planetary Quarantine Laboratory in Phoenix, Arizona. These spores were subdivided into a group suspended in ethanol and a group suspended in distilled water. The second spore crop was prepared in our own laboratory according to the following procedure. Spores were grown on TAM Sporulation Agar (Difco) supplemented with 20 ppm $MgSo_4$ and 80 ppm CaCl and were incubated at 41°C. After 48 hours, the spore crop was harvested by washing the growth off the agar surface with sterile distilled water. Any plates which had not obtained 90% sporulation in this period were discarded. The resulting suspension was cleaned by 6 or 7 centrifugations and distilled water washes. Following cleaning, spores were suspended in:

a. 95% ethanol and then stored at -5 to -10°C until use with subsequent dilutions made in 95% ethanol,

b. 95% ethanol and stored at -5 to -10°C, then resuspended in distilled water for use, or

c. distilled water and stored at 4°C until use with all dilutions made in distilled water.

3. Strip inoculation. All strips were inoculated by pipetting 0.02 ml (10^5 spores) onto the center of the strip. The inoculum was deposited as a drop; the drops were not spread mechanically. Inocula from water suspensions tended to remain in drop form while inocula from alcohol suspensions tended to spread into a thin film.

4. Equilibration. All strips were equilibrated for 20 to 24 hours in a laminar flow room (LFR) at 35 to 40% relative humidity (RH) and 23 ±1°C. Strips were protected from direct airflow but circulation of the air was permitted.

5. Strips in containers. Strips were placed in a thermal death time can (also equilibrated 20 to 24 hours in the LFR), one strip per can. In Experiment 1 the cans were closed in the laminar flow room. In Experiment 2 before closing in the LFR, specific quantities of filter paper, also equilibrated in the LFR for about one week, were added to the cans. Eight strips were held for a control in each experiment.

6. Heating. Cans were heated in an oil bath (115°C). Eight cans each were removed after 10, 20, 40 and 80 minutes of heating. Immediately after removal from the oil bath the cans were placed in cold water then cleaned and opened aseptically.

7. Processing. All strips were processed by NASA Standard Methods. Appropriate dilutions were made from the eluate. When high counts were expected 0.1 and 1.0 ml dilutions in single strength TSA were made; in instances where low counts were expected two 10.0 ml dilutions were plated using 1 1/2 strength TSA and the remaining eluate was plated with an equal amount of double strength TSA. When intermediate counts were anticipated, two 1.0 ml dilutions and two 10.0 dilutions

were plated.

RESULTS AND DISCUSSION

The results of the $D_{115°C}$-value determinations are summarized in Table 3.1. Representative survivor curves from these experiments are shown in Figures 3.1 and 3.2. The results of these experiments show three types of variation. First, there is considerable variation among duplicate experiments. Secondly the Phoenix spores exhibit larger D-values in water, whereas the Minnesota grown spores exhibit larger D-values in ethanol. Thirdly, the shape of the survivor curves of the Minnesota spores varies. At the present time, these variations cannot be adequately explained.

TABLE 3.1

$D_{115°C}$-Values of Spore Crops with Varying Histories

Test Number	Spore Source	Storage Medium	Suspending Medium	$D_{115°C}$-Value (minutes)
1*	Phoenix	Ethanol	Ethanol	16.5
2*	Phoenix	Ethanol	Ethanol	10.8
3	Phoenix	Water	Water	21.0
4*	Minnesota	Ethanol	Ethanol	50.1
5*	Minnesota	Ethanol	Ethanol	45.3
6	Minnesota	Ethanol	Water	28.9
7	Minnesota	Water	Water	14.3

* Tests 1 and 2, 4 and 5 are duplicate experiments.

The Minnesota spores stored in ethanol exhibited the largest D-values; however, both the spores stored in ethanol and suspended in ethanol and the spores stored in ethanol and suspended in water yielded

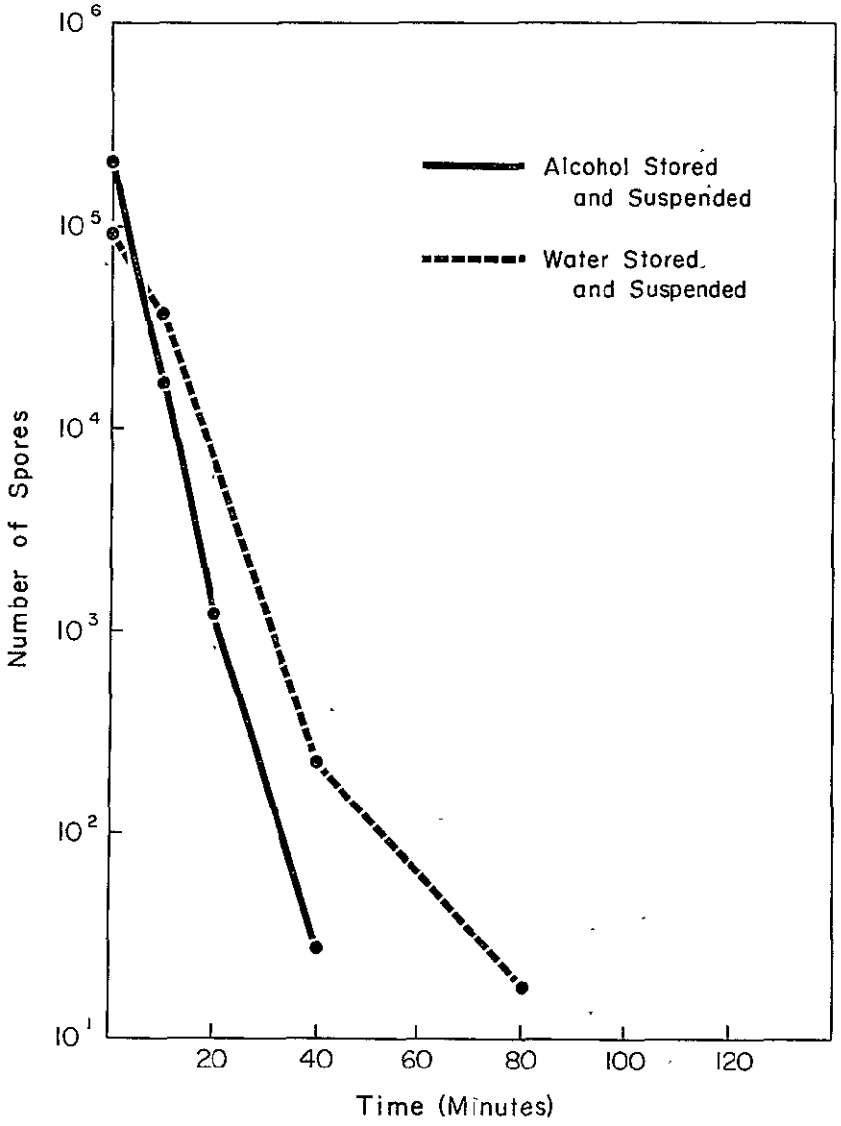

FIGURE 3.1

Dry heat survivor curves at 115°C for the Phoenix Laboratory strain of Bacillus subtilis var. niger spores.

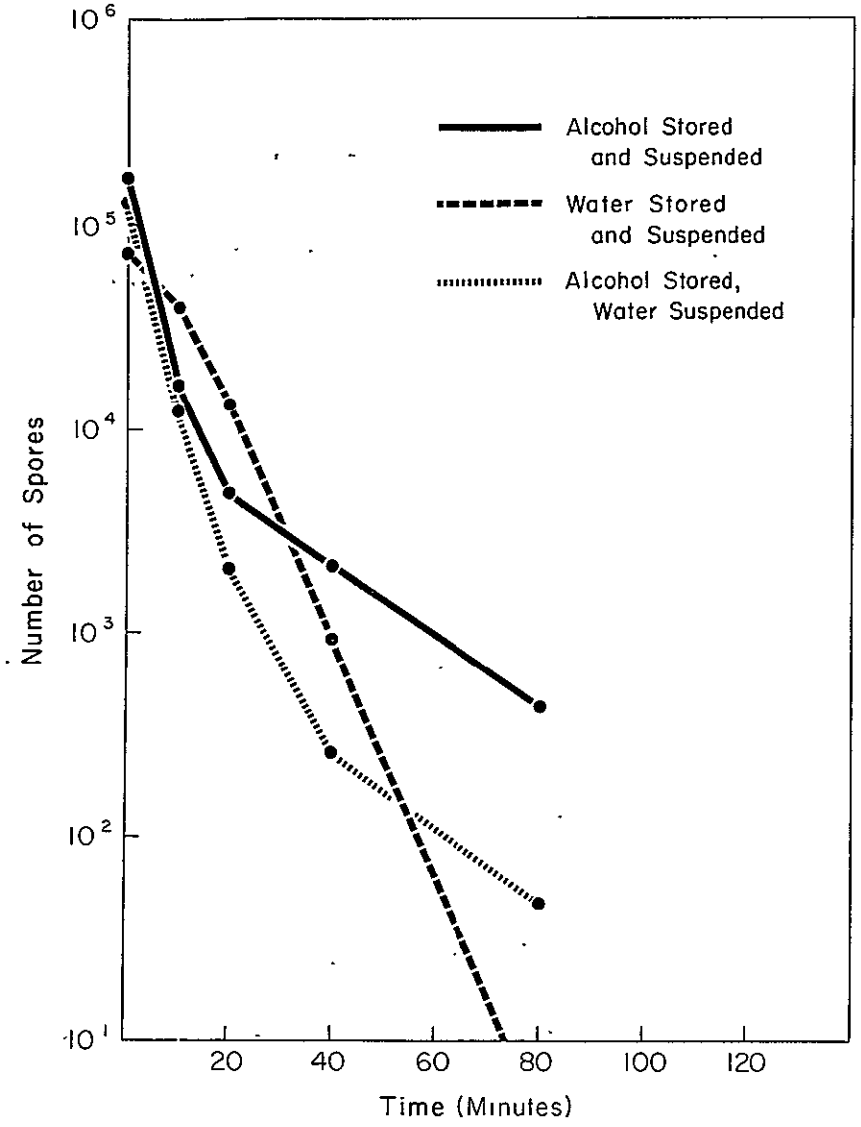

FIGURE 3.2

Dry heat survivor curves at 115°C for the University of Minnesota strain of Bacillus subtilis var. niger spores.

survivor curves typical of a spore suspension containing spores with different levels of heat resistance. In contrast to the shape of the curves for spores that have been in contact with ethanol, the Minnesota spores that were stored and suspended in water produced a linear survivor curve. Both Phoenix spore suspensions, those in ethanol and those in water, resulted in nearly linear survivor curves. A possible explanation for the phenomenon observed in these tests is that storage of spores in ethanol has the effect of making some spores more heat resistant, thereby creating a non-linear survivor curve. However, apparently this effect did not occur on the Phoenix spores where the survivor curve for the ethanol stored spores was a straight line.

It seems apparent that spores that see ethanol can have different D-values from spores of the same spore crop that see only water. The effects are apparently not consistent and probably there are other variables involved that have not yet been identified.

It appears at this time that these problems will have to be studied further to determine variations that occur in a specific laboratory; it also will be necessary to standardize intra-laboratory results through joint laboratory tests where personnel from each laboratory conduct simultaneous D-value determinations on spore crops prepared in two or more laboratories.

Evaluation of the results from the experiment where filter paper was added to the TDT cans is in progress.

CONCLUSIONS

The unexplained discrepancies in the results with the several

spore suspensions preclude any meaningful conclusions at this time. There are a multitude of factors which affect D-value determination and D-values can only be meaningful for highly explicit sets of circumstances.

FUTURE WORK

Studies to find out why spore crops vary and the role of ethanol in spore crop resistance levels and in survivor curve shapes will be continued. Side-by-side dry heat resistance studies will be conducted by personnel in our laboratories with personnel in other laboratories to standardize methodology for dry heat resistance studies.

THE EFFECT OF HUMIDITY, LOCATION, SURFACE FINISH AND SEPARATOR THICKNESS ON THE DRY HEAT DESTRUCTION OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES LOCATED BETWEEN MATED SURFACES

Project Personnel: D. W. Drummond, J. Haugen and I. J. Pflug - Division of Environmental Health

Project Contributors: F. B. Martin - Division of Biometry

INTRODUCTION

This is a continuation of the studies reported in the December, 1968, Progress Report which were directed toward quantifying and proving reproducibility of the preliminary data which was presented in Report #1. To add perspective to the study an experiment was carried out to measure the destruction rates in an open system of the same spores we are using in the mated system.

OBJECTIVE

The project will measure the thermal death parameters of *B. subtilis* var. *niger* spores in a mated surface system and will measure the effect of certain variations of the environment and of the surfaces on thermal destruction.

EXPERIMENTAL PROCEDURE

Mated System. The fundamental equipment and procedures were described in Report #1. Photographs of the heat block and the test system were shown in that report.

The same lot of spores is being used in all tests. The spores were produced in our laboratory from spores supplied by the CDC Phoenix

laboratory. The spores were grown on supplemented TAM agar at 41°C, washed and stored in ethanol at approximately -10°C. After several months of storage, the spores were rinsed and resuspended in sterile distilled water. The suspension was subdivided into small bottles and stored frozen at approximately -10°C. A bottle of spores is thawed prior to an experiment; the spores remaining after the experiment are discarded. In this procedure all spores are subjected to the same handling history except for the length of frozen storage.

Several other procedures have been adopted to improve the accuracy and reliability of the experimental procedure.

1. Experiments are fully carried out in one day and are replicated on at least two different days.
2. All experimental data are corrected for temperature variation.
3. A semi-blind system is used for colony counting; a double blind system was tested but was too cumbersome.
4. The following operations have been randomized:
   a. assignment of 6 x 8" plates to treatments,
   b. order of treatment,
   c. order of processing strips, and
   d. order of plate counting.

A check was made of spore transfer from the base plate to the cover plate. The number of spores transferred from the base plate to the non-analyzed cover plate of the mated surface system was evaluated and found to be less than 3% in all cases. This is well inside the limits of our experimental error.

Humidity conditioning of the spores after they have been dried onto the test surface is carried out in a plastic glove box. In the box, the relative humidity is controlled at <2% or at 90% using wetted silica gel. A small electric fan in each box prevents vapor stratification. The boxes are at the ambient temperature of about 25°C. The relative humidity in the boxes is verified using a Honeywell W6IIA relative humidity indicator.

For treatment the experimental apparatus is placed in a refrigerator or in a humidified incubator where the dew point is 5°C or 21°C giving treatment humidities of 0.34% and 1.1% respectively at 125°C.

Open System. To add perspective to the results, the same spores used in the mated system tests were tested in an open system. The open system used a gravity convection hot air oven, Blue M #OV-12A. One by two inch stainless steel strips are hung on a rack and inserted through small doors to avoid disturbing the temperature of the oven. (See Figures 4.1 and 4.2.) A diffuser plate was installed inside the oven to help

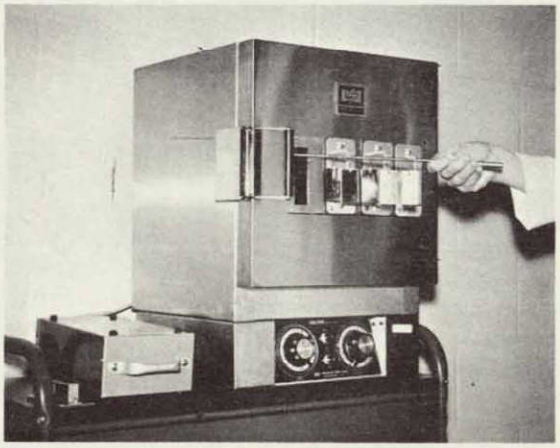

FIGURE 4.1 Open system test oven showing insertion of sample carrying rack.

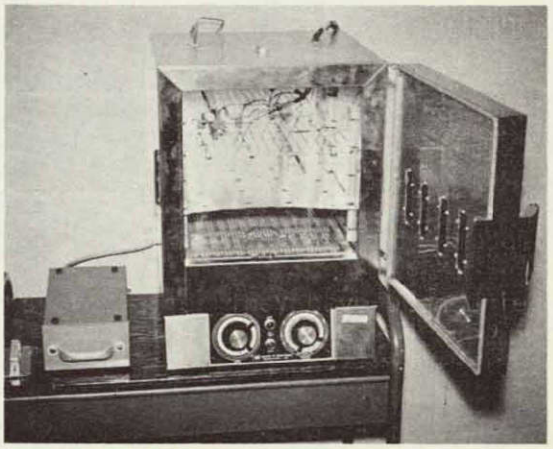

FIGURE 4.2 Test oven showing diffuser plate added to decrease temperature variations.

minimize temperature variation across the oven. A variable transformer and a thermistor temperature controller were added to the oven to decrease overshoot and to give accurate control. The test procedures and treatment conditions for the open system were identical with those used in the mated system except that a period of about 8 minutes elapsed after removal from the conditioning environment and before insertion into the oven.

The treatment conditions for open and mated systems are summarized in Table 4.1.

TABLE 4.1

Summary of Experimental Conditions

OPEN SYSTEM EXPERIMENT				
Conditioning Relative Humidity	Treatment Relative Humidity	Treatment Time (min)	Treatment Temp.	Number of Replicate Experiments
<2%(L) 90%(H)	0.34%(D) 1.1%(W)	10 30 50 70	125°C	2
MATED SYSTEM EXPERIMENT				
<2%(L) 90%(H)	0.34%(D) 1.1%(W)	15 40 65 90	125°C	5

RESULTS AND DISCUSSION

Presently available results are summarized in Figures 4.3, 4.4, and 4.5 and in Table 4.2. Further information will be available when the data analysis is complete.

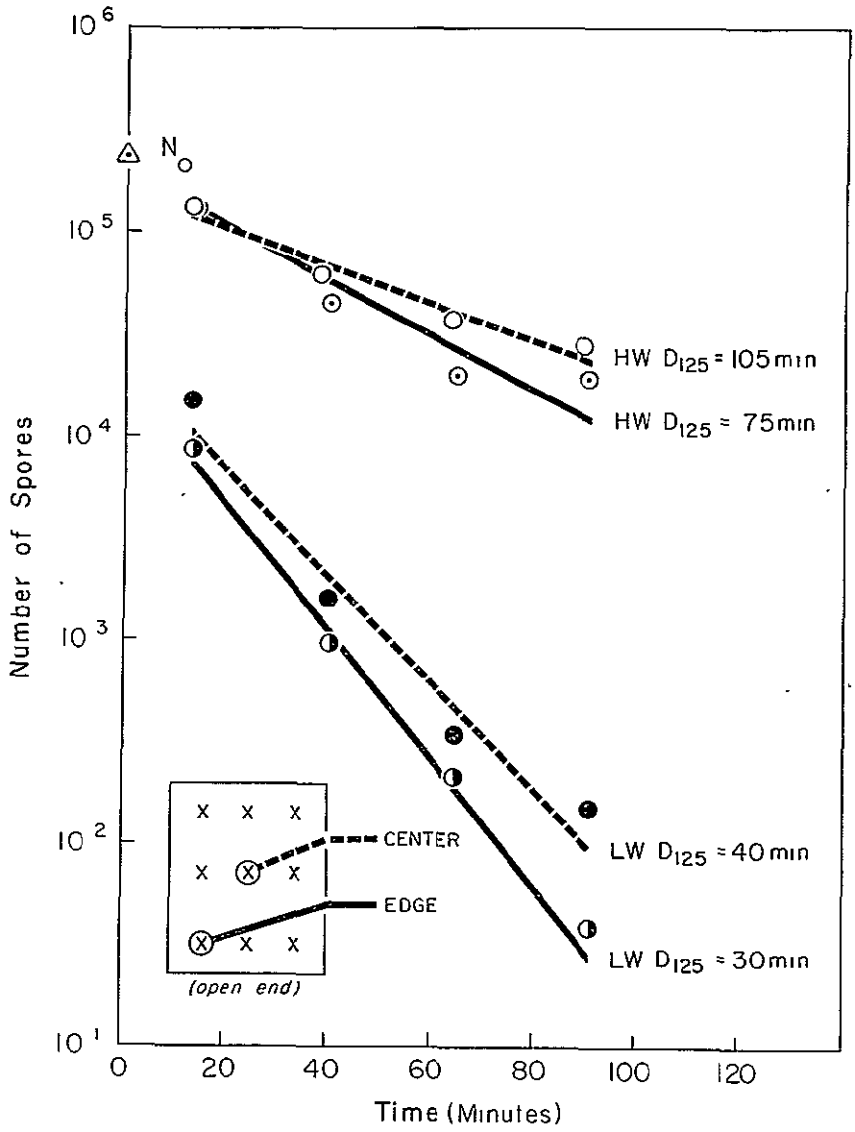

FIGURE 4.3

Dry heat destruction curves for Bacillus subtilis var. niger spores on stainless steel in a mated surface area system.
(HW = conditioned at 90%RH, treated at 1.1%RH;
LW = conditioned at <2%RH, treated at 1.1%RH.)

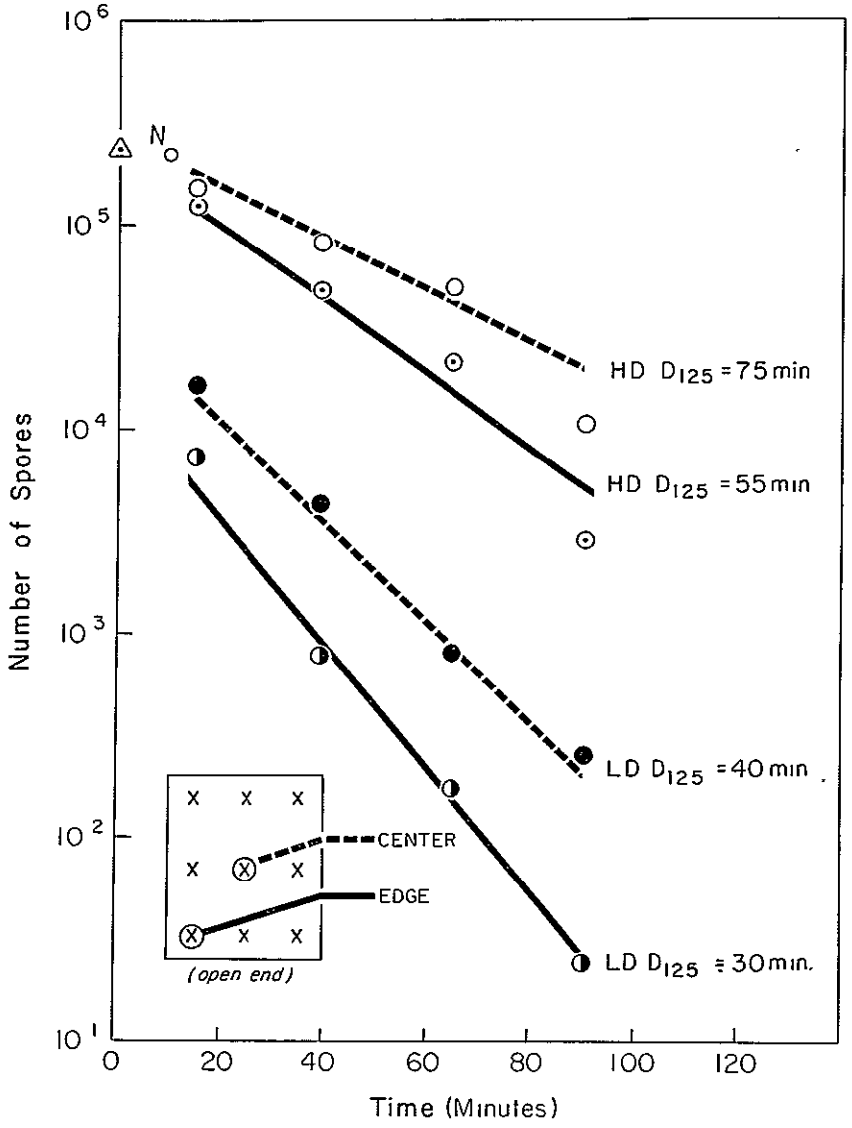

FIGURE 4.4

Dry heat destruction curves for Bacillus subtilis var. niger spores on stainless steel in a mated surface area system.
(HD = conditioned at 90%RH, treated at 0.34%RH;
LD = conditioned at <2%RH, treated at o.34%RH.)

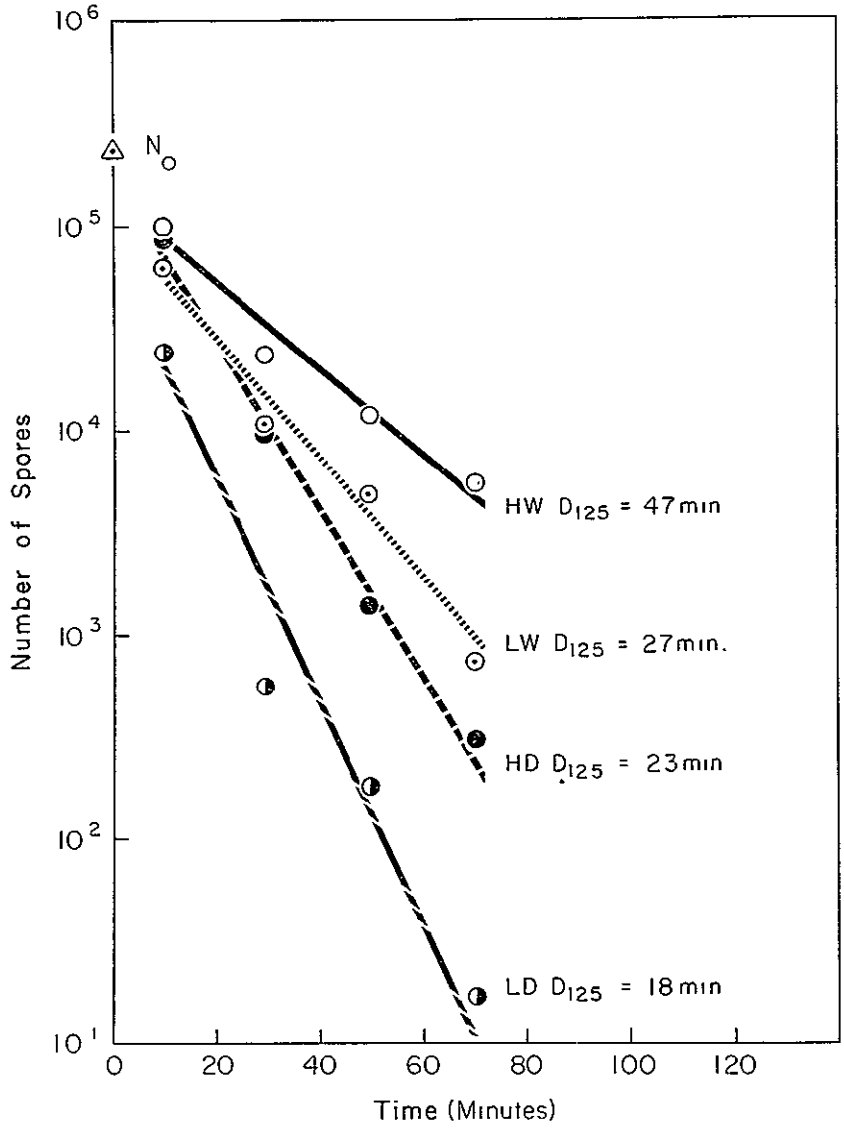

FIGURE 4.5

Dry heat destruction curves for Bacillus subtilis var. niger spores on stainless steel in an open system.
(LD = conditioned at <2%RH, treated at 0.34%RH;
HD = conditioned at 90%RH, treated at 0.34%RH;
LW = conditioned at <2%RH, treated at 1.1%RH;
HW = conditioned at 90%RH, treated at 1.1%RH.)

TABLE 4.2

Summary of D-values in Minutes for Spores on Surfaces and in Mated Surface Areas

Humidity Code*		LD	LW	HD	HW
Conditioning RH at 26.7°C		<2%	<2%	90%	90%
Treatment RH at 125°C		0.34%	1.1%	0.34%	1.1%
OPEN SYSTEM		18	27	23	47
MATED SYSTEM	1" from exposed edge	30	30	55	75
	at center of 6" x 8" sheet with one exposed edge	40	40	75	105

*Conditioning RH
L = < 2%
H = 90%

Treatment RH
D = 0.34%
W = 1.1 %

The open surface system gives smaller D-values than does the mated system. It also shows relatively less effect of conditioning humidity.

The delay between removal from the conditioning box and insertion into the treatment oven was about 8 minutes in all cases. Campbell (1969)[a] has found that when dry spores are subjected to an RH of 43% they absorb water so rapidly they are about two-thirds equilibrated to the new condition in 8 minutes; therefore, the open surface experiments are only indicative of an equilibration effect. In any case, it is clear that the D-value of spores on surfaces is very sensitive to the water content of the air contacting them during both conditioning and treatment; the water con-

[a] Campbell, J. E., 1969. Quarterly Progress Report #16 (April). Environmental Control Administration, Public Health Service, Cincinnati, Ohio.

tent of the air must be measured and controlled during dry heat experiments.

In the mated surface experiments it was observed that when the samples were equilibrated at the low conditioning humidity, changing the treatment humidity produced little or no effect regardless of whether the spores were located at the edge or at the center of the surface. The spores at the center of the plate showed a larger D-value: 40 minutes compared to 30 minutes at the edge.

The results suggest that when the experimental packages containing spores equilibrated at a low humidity are heated, the rate of moisture diffusion from the edge is relatively small; at least it is low enough so as to appear independent of the ambient vapor pressure. However, the difference in D-value between the center and the edge suggests that there is a measurable difference between the water content at the center and at the edge.

Based on the 90 minute treatment, it is probable that treatment humidity as well as position affects thermal destruction of spores equilibrated at a high humidity. The D-value at the center was about 25% greater than at the edge for both treatment humidities.

In the mated surface experiments it is clear that changes in either conditioning or treatment humidity will cause changes in the D-value. At present, water content appears to be the only variable that can bring about these changes in D-value since spore variables are held constant. It is generally assumed that a straight line semi-logarithmic survivor curve only results if conditions are non-changing and the spores are homogeneous. If water conditions are changing then it might be anticipated that the survivor curves will not be straight lines on semi-logarithmic paper but instead will be curves. However, when spores on plates conditioned under

different humidities are treated under identical conditions, the survivor curves do not converge at times of less than 90 minutes even for spores at the edge locations. When plates that are conditioned in the same relative humidity were subjected to different relative humidities during treatment at times of 90 minutes or less, no divergence occurs. A limited amount of data at long exposure times (up to 6 hours) does not contradict these observations.

CONCLUSIONS

The conclusions apply only to our system of Bacillus subtilis var niger spores with a history of alcohol storage treated in specific open and mated surface systems.

Open System

1. The humidities of the atmospheres surrounding spores on open surfaces prior to and during treatments affect the D-value and must be considered as experimental variables of the system.

Mated System

1. The distance of spores from the open edge of the mated surface system has an effect on the D-value.

2. When spores are conditioned at a low (<2%) relative humidity, a change of treatment humidity from 0.34% to 1.1% has no effect on the D-value.

3. When spores are conditioned at 90% relative humidity, a change of treatment humidity from 0.34% to 1.1% may have an effect on the D-value.

Both Systems

1. Conditions that tend to increase the water content of the spores tend to increase the D-value.

2. Conditions that tend to increase the water content of the spores tend to raise the intercept of the D-value line on the zero time axis.

FUTURE WORK

Open System. Further open system studies should be carried out in an oven which produces a controlled uniform air movement across the treatment surfaces. In view of Campbell's (1969)[a] data a system should be developed such that the samples are not removed from the conditioning environment until treatment begins.

Mated System. In the mated surface system there appear to be two areas that should be explored further as far as our model system is concerned. These are equilibration at an intermediate humidity (somewhere in the range 30 to 40%RH) and a test condition where the heating is carried out in dry nitrogen which will have a humidity much lower than those that have been studied to date. Tentatively it appears desirable to carry out additional experiments in which

1. the spores are equilibrated at a relative humidity of 2% and tested in the dry nitrogen atmosphere,
2. the samples are conditioned at a relative humidity in the range of 30 to 40% and tested in dry nitrogen and in an atmosphere with a relative humidity of 0.34% and 1.1%, and

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[a] Campbell, J. E., 1969. op. cit.

3. the spores are conditioned at 90%RH and tested in the dry nitrogen atmosphere.

Previous studies have suggested that surface finish has an important effect. The role of surface finish will be investigated at one humidity condition for two types of surface finishes.

The z-value is an important parameter in dry heat sterilization processes; z-value will be determined for spores in our model mated surface area system for high and low moisture conditions.

Several moisture conditions in the mated surface system will be examined at long treatment times where during heating the microbial population will undergo a reduction from, for example, 10^5 to 10^2 microorganisms. This experiment will give more information concerning the nature of the dry heat destruction rate curve for Bacillus subtilis var. niger.

DETECTION OF LOW LEVELS OF MICROBIAL CONTAMINATION ON SURFACES BY CHEMICAL APPROACHES

Project Personnel: Velta Goppers and H. J. Paulus -
Division of Environmental Health

INTRODUCTION

Studies to develop chemical methods for detecting microorganisms on surfaces and to differentiate between dead and live microorganisms are in progress.

In previous Progress Reports studies were focussed on detection of microorganisms on surfaces, while in recent weeks studies have moved on to the problem of differentiation between live and dead microorganisms.

OBJECTIVE

The objective of these studies is to be able to use chemical methods to:

1. determine the number of microbial cells present on a surface, and
2. determine the relative percentage of living and dead cells.

EXPERIMENTAL PROCEDURE

Thin layer chromatography as described in the two previous reports is used to separate the compounds existing in live microorganisms and also after the thermal destruction of the microorganisms in order to determine the chemical changes in the molecule and detect the compounds which would indicate a dead microorganism. At present, the experiments are conducted under regular laboratory conditions including exposure to

natural, uncontrolled relative humidity and other physical factors.

The material used was E. coli in growth phase prepared on Bacto-tryptone agar plates. One colony of E. coli was transferred to 0.5 ml of distilled water and immediately spotted in microliter quantities on previously prepared thin layer plates, as described in Progress Report #1, and dried in air for 10 minutes. A total of ten plates were prepared in a similar way with E. coli washed in distilled water in order to separate the salts present from the growth medium. The plates were then divided into five groups of four each. The first group was immediately transferred to chromatography chambers for developing as described in the December, 1968, Progress Report; the rest of the groups were exposed for 15, 30, 60 or 180 minutes at 125°C to study thermal destruction.

Heating was carried out in a dry heat oven. After the indicated time each group was removed from the oven and cooled in air for ten minutes. A special plate was prepared with standard chemical compounds. All plates were developed chromatographically in the manner described in previous reports. After developing, the plates were removed from the chromatography chamber and dried in air for 15 minutes, then visually examined in ultraviolet light. The experiment was repeated ten times.

RESULTS AND DISCUSSION

Each plate containing microorganisms exposed to heat was examined along with the unheated control plates. The chromatographic patterns revealed that after heating purine and pyrimidine compounds appear. At this point in the project, the results are not reproducible. After repeated experiments we conclude that the thermal destruction compounds appear after 15 minutes of heating at 125°C when the relative humidity is about

45% but do not appear after heating for the same length of time and at the same temperature when the relative humidity is 98%. The inability to obtain reproducible results may be due to variation in the initial water content of the microbial cells. Microorganisms absorb and desorb water very rapidly; the quantity of water absorbed or desorbed is a function of the relative humidity. Since these experiments are carried out in a laboratory where the relative humidity is known to vary, it is possible that changes in the ambient humidity are responsible for the variation. Plans are underway to carry out these studies in a controlled humidity environment.

One and three hour heating times produced purine and, in some cases, pyrimidine compounds. It is obvious that factors other than relative humidity are involved which must be standardized or correction values established. An example is the thickness of the glass plates or the stainless steel strips.

The results, although not reproducible under present conditions of varying relative humidity, in two runs were positive and are quite important. This experiment with ten plates, replicated ten times and analyzed for the derivatives, especially in the 15 and 30 minute heating groups established the fact that the DNA chain is attacked by thermal treatment and purine-adenine is separated first, followed by pyrimidine-thymine. It is known that these compounds are coupled with only two hydrogen bonds and are the weakest link in the DNA chain.

CONCLUSIONS

The preliminary results indicate that the method used with thin layer chromatography offers a procedure to separate nucleotides which are

produced from thermal denaturation of the DNA chain in microorganisms. It seems probable that under closely controlled conditions it may be possible to determine the crucial dead/live microorganism point.

FUTURE WORK

Future work will involve completion of the first phase of the research project. An answer to the question "How many bacteria are on a surface?" will be obtained by using the microscope photometer in connection with the thin layer chromatography procedure. The microscope photometer has been received and is now being adjusted by the manufacturer.

It is also planned to continue work on the differentiation between live and dead bacteria. The temperature and humidity for the thermal destruction study will be standardized. Use of thin layer chromatography coupled with ultraviolet methods and infrared spectroscopy will be utilized to separate and identify the resulting compounds. At the same time new methods or modifications of current methods will be developed according to our needs.

PUBLICATIONS AND PRESENTATIONS

DURING PAST SIX MONTHS

Presented at Meetings and Seminars

1. Drummond, D. and I. J. Pflug; "Effect of Humidity on the Dry Heat Destruction of Bacillus subtilis var. niger Spores on Surfaces"; presented at the Annual Meeting of the American Society for Microbiology; Miami Beach, Florida; May, 1969.

2. Goppers, V.; "Toxic Effects of Allergenic Macromolecular Compounds on Guinea Pigs and Mice"; presented at the American Industrial Hygiene Conference; Denver, Colorado; May, 1969.

3. Pflug, I. J.; "Dry Heat Destruction Rates for Microorganisms on Open Surfaces, in Mated Surface Areas and Encapsulated in Solids of Spacecraft Hardware"; presented at the XII Plenary Session of the Committee on Space Research; Prague, Czechoslovakia; May, 1969.

4. Vesley, D., G. Smith and I. J. Pflug; "Effect of Relative Humidity on Survival of Bacillus subtilis var. niger Spores at 22 and 45°C"; presented at the Annual Meeting of the American Society for Microbiology; Miami Beach, Florida; May, 1969.

5. Vesley, D., G. S. Michaelsen and M. M. Halbert; "Effect of Air Velocity on Biocontamination in a Laminar Crossflow Room"; presented at A^2C^2 Annual Technical Meeting; New York, New York; May, 1969.

Publications in the Open Literature

1. Vesley, D., G. S. Michaelsen and A. A. Levitan; "The Application of Laminar Flow Rooms to Patient Isolation"; Germ Free Biology; Plenum Press; New York, New York; 1969.